

University of Groningen

Engineering covalent oligomers of the mechanosensitive channel of large conductance from *Escherichia coli* with native conductance and gating characteristics

Folgering, JHA; Wolters, JC; Poolman, B

Published in:
Protein Science

DOI:
[10.1110/ps.051679005](https://doi.org/10.1110/ps.051679005)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2005

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Folgering, JHA., Wolters, JC., & Poolman, B. (2005). Engineering covalent oligomers of the mechanosensitive channel of large conductance from *Escherichia coli* with native conductance and gating characteristics. *Protein Science*, 14(12), 2947-2954. <https://doi.org/10.1110/ps.051679005>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Engineering covalent oligomers of the mechanosensitive channel of large conductance from *Escherichia coli* with native conductance and gating characteristics

JOOST H.A. FOLGERING, JUSTINA C. WOLTERS, AND BERT POOLMAN

Department of Biochemistry, Groninger Biomolecular Sciences and Biotechnology Institute, and Materials Science Centre^{plus} (MSC^{plus}), University of Groningen, 9747 AG, Groningen, The Netherlands

(RECEIVED June 30, 2005; FINAL REVISION September 23, 2005; ACCEPTED September 27, 2005)

Abstract

To obtain a gene construct for making single substitutions per channel and to determine the quaternary structure of the mechanosensitive channel MscL from *Escherichia coli*, covalent oligomers (monomer to hexamer) were engineered by gene fusion; up to six copies of the *mscL* gene were fused in tandem. All the multimeric tandem constructs yielded functional channels with wild-type conductance and dwell times. Importantly, only the covalent pentamer opened at the same relative pressure (compared to the pressure required to open MscS) as the wild-type MscL channel. The in vivo data strongly suggest that pentameric MscL represents the functional state of the channel.

Keywords: MscL; oligomeric structure; covalently linked oligomer; structure/function studies; membrane proteins

Mechanosensitive (MS) channels play a critical role in the survival of microorganisms upon a decrease of the external osmolarity. The biochemically and biophysically best characterized MS channel is the Mechanosensitive Channel of Large conductance (MscL) from *Escherichia coli*. MscL gates in response to tension in the cell membrane, which is caused by the influx of water into the cell upon osmotic downshift. After the opening of MscL, osmolytes and excess water leave the cell, which prevents cell lysis (Poolman et al. 2002).

When MscL activity was first described and the corresponding gene identified in *E. coli* (Sukharev et al. 1994), it was clear that the channel, with a conductance of 2.5–4 nS, had to be an oligomer of the relatively small (136 amino

acids) polypeptide. Moreover, octyl- β -glucopyranoside-solubilized MscL migrated through a gel filtration column as a complex of 60–80 kDa rather than the expected 17 kDa predicted for a monomeric species. On the basis of these experiments, a tetrameric channel was proposed (Hase et al. 1995). However, the exact number of subunits per functional channel has been a matter of discussion ever since.

The response of MscL to sustained membrane tension is different when a small number of channels (an activity burst is followed by inactivation) instead of a large number of channels (sustained or increasing channel activity) is present in a patch. From these observations it has been suggested that the channels assemble in the membrane upon application of mechanical force (Hase et al. 1995), but conclusive experiments have never been presented. Studies in which wild-type MscL and a dimeric version of the polypeptide were subjected to cross-linking resulted in the proposition that the functional MscL unit consisted of six subunits (Blount et al. 1996a, Hase et al. 1997). This hexameric conformation

Reprint requests to: Bert Poolman, Department of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG, Groningen, The Netherlands; e-mail: B.Poolman@rug.nl; fax: +31-50-3634165.

Article and publication are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.051679005>.

was also supported by a two-dimensional crystallization/electron microscopy study (Saint et al. 1998).

A major breakthrough came when the structure of MscL from *Mycobacterium tuberculosis* was determined by X-ray crystallography (Chang et al. 1998). This not only gave insight into the pore structure and potential gating mechanism but also showed that the *M. tuberculosis* channel consists of five subunits. Subsequently, the quaternary structure of MscL from *E. coli* was examined by cross-linking, size exclusion chromatography (SEC), and analytical ultracentrifugation, using the wild-type, dimeric, and trimeric tandem constructs. Although the reported analytical ultracentrifugation, cross-linking, and SEC data are not unambiguous, this work is most consistent with the proposal that MscL from *E. coli* also forms a channel of five subunits (Sukharev et al. 1999). More recently, an electron microscopic study on a homogeneous population of MscL particles confirmed a pentameric structure (Becker et al. 2004).

Although there is no evidence that the oligomeric structure of MscL differs from species to species, such information does exist for other membrane-bound protein complexes, e.g., the F_1F_0 -ATP synthase (in yeast it contains 10 *c* subunits; in chloroplasts, 14; and in *Ilyobacter tartaricus*, 11) (Stahlberg et al. 2001). Recently, pore-forming toxins were identified in *Staphylococcus aureus* that vary in their subunit composition. These pores consist of subunits of LukF and Hlg2 in either a 3:4 or a 4:3 ratio and are thought to play a role in hemolysis of human erythrocytes (Sugawara-Tomita et al. 2002). Both these examples demonstrate that oligomeric structures of proteins (or protein complexes) may differ between species and even within a single species.

Homo-oligomeric structures complicate the analysis of the contribution of individual subunits in the protein function, because it is not possible to engineer single mutations per functional unit. To overcome this problem, Liman et al. (1992) covalently linked oligomers of the mammalian K^+ channel. Here, we present the engineering of a covalently linked pentameric MscL channel, which offers unique possibilities for further studies of channel gating by making single or multiple amino acid substitutions or introducing pairs of fluorophores at specific positions in the channel. The covalently linked oligomers also provide in vivo evidence for the pentameric structure of MscL from *E. coli*.

Results

Engineering strategy and expression of the tandem constructs

To produce covalent oligomers of MscL, a series of plasmids was constructed with one, two, three, four, five, or six

mscL genes fused in tandem (Fig. 1). The genes were placed under control of the tightly regulated arabinose promoter (P_{BAD}) (Guzman et al. 1995). The gene constructs were designed with unique restriction sites between the individual genes, which allow easy engineering of mutations by exchange of one or more gene fragments. The added base pairs yield insertion of two amino acids between the individual MscL units. The linkers were kept short because previous work had shown that di- and trimeric tandem constructs are active with linkers of only a single amino acid (Blount et al. 1996a, Sukharev et al. 1999). The engineering strategy results in N-terminal extensions for the mono-, di-, and trimeric constructs as indicated in Figure 1. Finally, the last gene of each construct was extended with a sequence specifying a 10-histidine tag.

On the basis of expression screening (comparing different L-arabinose concentrations, induction times, and growth temperatures) with the covalent pentameric *mscL* gene (data not shown), L-arabinose at 0.0025% (w/v) and two hours of induction at 30°C were used for further studies with all the tandem constructs. An immunoblot was used to visualize the protein levels as shown in Figure 2. Except for the tetrameric fusion, translation of the constructs started from the ATG codon at the beginning of the multiple cloning site in pBAD, resulting in different N-terminal extensions relative to wild-type MscL for the mono-, di-, and trimeric constructs (Fig. 1A). As shown in Figure 1B, the insertion of the *mscL2* gene into the *SacI* site resulted in a single base-pair frame shift, relative to the beginning of the multiple cloning site in pBAD at 11 nt downstream of the ATG translation initiation codon. Most likely, the tetrameric construct is translated from the second ATG and starts, like the wild-type, pentameric and hexameric constructs of MscL, with the sequence MSII (Fig. 1B). The final level of expression of the tetrameric construct was lower than that of the other constructs (Fig. 2), probably because the distance between the Shine-Delgarno sequence and translation initiation of *mscL2* is too long. It is also possible that an alternative weaker Shine-Delgarno sequence closer to the *mscL2* ATG is used (Fig. 1B).

Longer exposures of the immunoblots showed that the anti-his antibody had also reacted with some smaller-sized proteins or protein fragments (data not shown). Most of these bands were also present in the empty plasmid control and are therefore aspecific. However, some of the smaller bands were related to the expression of a particular tandem construct, indicating that some degradation had taken place, possibly during the preparation of the membrane vesicles.

All tandem constructs encode functional channels

Patch clamp experiments with spheroplasts expressing the different MscL tandem constructs revealed that all

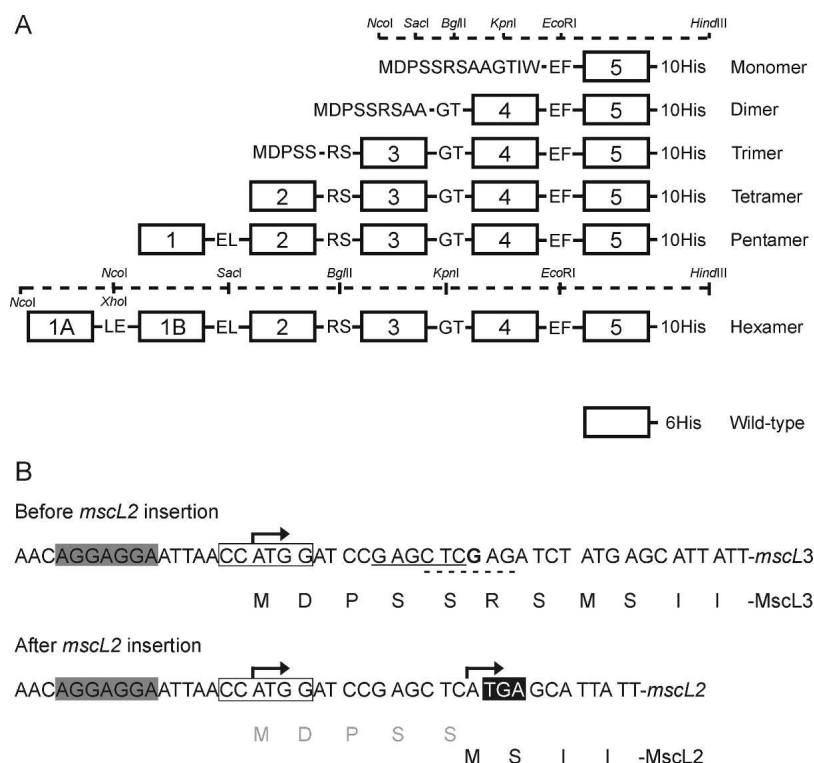


Figure 1. (A) Schematic representation of the multimeric MscL constructs. *Top* dashed line indicates relevant restriction sites in the multiple cloning site of the pBADmyc-HisB plasmid before cloning. The α -amino acid sequences of the linkers of the multimeric tandem constructs are indicated. The monomeric, dimeric, and trimeric tandem constructs each start at the ATG of the NcoI restriction site in the plasmid and therefore contain N-terminal extensions as indicated. Each box represents the full sequence of one wild-type MscL from *E. coli*; the numbers *inside* the boxes indicate the number of the MscL units (Tables 1, 2). The 10 his-tag at the C terminus of the proteins is also shown. The second dashed line indicates the position of the relevant restriction sites in the multiple cloning site of the pBAD plasmid after cloning of the DNA fragments. Positions of the relevant restriction sites in the pentameric tandem construct are shown *above* the second dashed line; additional changes in restriction sites for the hexameric tandem construct are indicated *below* the second dashed line. For comparison, wild-type MscL is also shown. (B) Ribosome-binding site (indicated in gray) and translation initiation sites (indicated with arrows) before (*top*) and after (*bottom*) insertion of *mscL2*, using the SacI restriction site (solid underline). The insertion of *mscL2* in SacI-BglII removes the guanine (bold) from the XhoI restriction site (dashed underline), resulting in a frameshift relative to the ATG of the NcoI restriction site (boxed). Translation from that ATG would result in a 5- α -amino acid peptide (indicated in gray) as the sixth triplet corresponds to a stop-codon (indicated as black box). Most likely, the tetrameric MscL fusion is expressed from the second ATG, which represents the start of the wild-type *mscL* gene. The sequences of the peptides resulting from translation at the indicated translation initiation sequences are shown *below* the DNA sequence.

covalent oligomers formed active mechanosensitive channels with a unitary conductance of ~ 3.0 nS (Fig. 3). A typical full electrophysiological recording of a spheroplast expressing wild-type MscS and the monomeric MscL construct is shown in Figure 3A. As the pressure in the pipette was increased, initially only opening (and closing) of MscS was observed. At ~ 1.7 times the pressure at which two simultaneous MscS openings were observed, the first full opening of MscL was recorded (indicated by an asterisk). In the empty plasmid control, indicated by 0 in Figure 3B, only MscS activity was observed. The number of channels observed per patch was generally between one and five for all the multimeric constructs except for the tetrameric fusion, where in a number of patches no

channel activity was observed, which is in accordance with the lower expression level (Fig. 2). Dwell times and substate preference of the oligomeric tandem channels were found not to differ significantly from those of the WT channel (data not shown). This is in contrast to previous findings (Sukharev et al. 1999), where it was reported that dimeric and trimeric tandems with one -amino acid linkers showed a slower open-to-closed transition. It seems that the two amino acid linkers used in our constructs were sufficient to minimize the constraints of covalently linking subunits, resulting in opening and closing characteristics similar to that of the wild-type MscL.

Next, giant spheroplasts of *E. coli* PB104 were used to examine the pressure at which the oligomeric

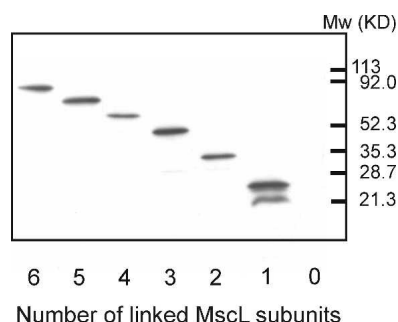


Figure 2. Immunoblot of *E. coli* PB104 membranes expressing the different MscL tandem constructs. The double band observed in the lane marked "1" is typical for monomeric (wild-type) MscL (Sukharev et al. 1994; Hase et al. 1997). All lanes are marked according to the number of MscL units in the construct.

tandem channels first opened fully relative to the pressure at which MscS opened. The pressure ratio of the monomeric construct was 1.69 ± 0.10 , which is comparable to the ratio found for wild-type MscL (1.64 ± 0.08) (Yoshimura et al. 1999). The pressure ratios for the di-, tri-, tetra-, and hexameric tandem constructs (2.61 ± 0.10 , 2.75 ± 0.13 , 3.30 ± 0.12 , and 3.33 ± 0.10 , respectively) were significantly higher and tended to increase with the number of linked subunits (Fig. 4). Importantly, the pressure ratio of the pentameric tandem construct (1.60 ± 0.09) was similar to that of the wild-type MscL, strongly suggesting that it represents the native oligomeric state.

Discussion

The pentameric construct results in channels with wild-type behavior

Based on the relative pressure ratios for full channel opening (Fig. 4), it can be concluded that the covalently-linked pentameric construct behaves similar to the wild-type MscL channel. The higher pressure ratio required for the opening of the covalent dimeric, trimeric, tetrameric, and hexameric constructs may be explained from the way these polypeptides form functional channels (Fig. 5). If it is assumed that one functional channel comprises of five subunits, one or more nonparticipating subunits must lie at the periphery of the channel in the case of the covalent dimeric, trimeric, tetrameric, and hexameric constructs, as originally suggested by Sukharev and colleagues (Sukharev et al. 1999). We propose that in the dimeric, trimeric, and hexameric constructs, one of the linked subunits is not participating in channel formation and is located at the periphery of the channel (Fig. 5B,C,F). For the tetrameric construct, it would imply that three subunits are not participating in channel formation (Fig. 5D). It is actually surprising that these constructs form

functional channels, with relatively little detrimental effects of the nonparticipating subunits. The peripherally-located subunits, however, do hinder the opening of the channel, as can be inferred from the increased pressures needed for initial MscL gating (Fig. 4). This implies that the presence of subunits, which do not participate in the formation of the pore, requires additional energy for

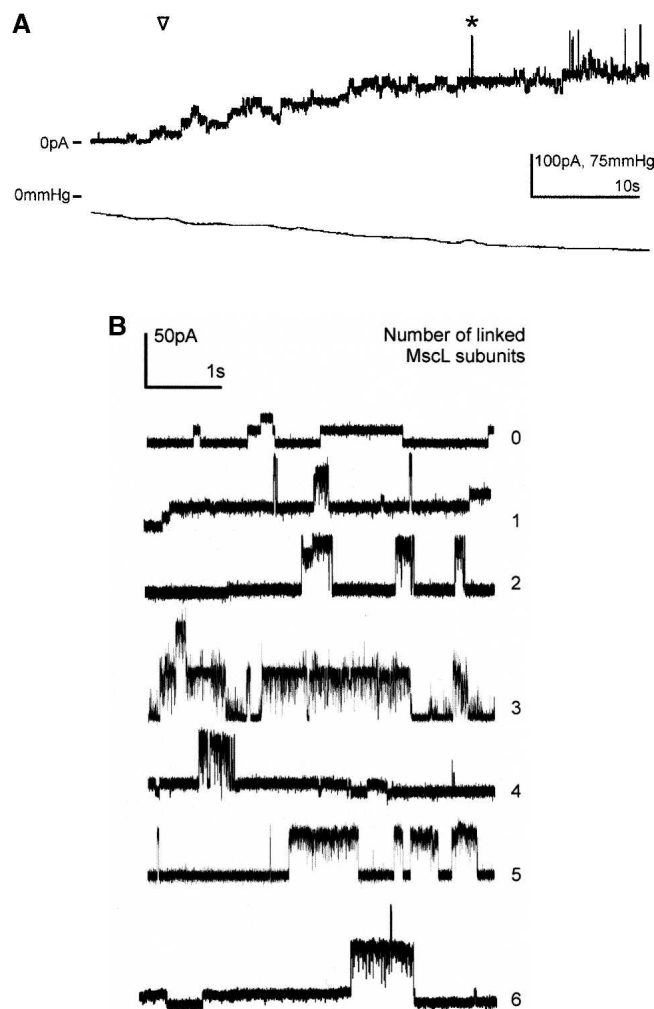


Figure 3. (A) Full electrophysiological and pressure recording of a spheroplast from *E. coli* PB104 expressing the monomeric construct, showing both MscS and initial MscL activity. Initial current and pressure are indicated. The pressure was gradually increased and the pressure ratio between MscS and MscL could be determined. ∇ , indicates first opening of MscS; *, indicates first opening of MscL. (B) Typical electrophysiological recordings of spheroplasts from *E. coli* PB104 expressing the different multimeric constructs. Patches were not saturated, but tension was set to a level where only one or two channels were activated. Time and current scale bars for all traces are indicated at the top. All traces are marked according to the number of MscL units in the construct as in Figure 2. The trace marked "0" corresponds to *E. coli* PB104 (containing the empty pBADmyc-HisB control plasmid), that is, the MscL null mutant in which MscS activity can still be observed. All recordings were performed at a pipette voltage of 20 mV.

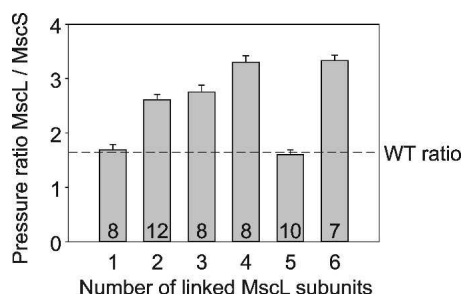


Figure 4. Pressure ratios of MscL/MscS. For each of the tandem constructs, the pressure required to fully open the first MscL channel was determined relative to the pressure at which the first two or more MscS channels opened simultaneously in the same patch (Blount et al. 1996b, 1999). The ratio of the wild-type MscL, as determined by Yoshimura et al. (1999), is indicated as a dashed line for comparison. The bars are marked according to the number of MscL units in the construct as in Figures 2 and 3A. The number of patches analyzed is indicated inside the bars. Error bars indicate SEM.

the cooperative opening of the channel. The linkers between the subunits may also influence the pressure ratios by restricting the channel flexibility. However, we feel that this is not likely as the pentameric tandem behaves like wild-type MscL.

It could be argued that the covalent hexameric tandem construct is not in the conformation proposed in Figure 5F, but in a conformation in which all six subunits participate in channel formation. In that case, the sixth subunit would lead to a 1.2-fold increase of the circumference of the channel and possibly a 1.2-fold increase in the pore diameter as compared to the suggested pentameric structure. The conductivity of such a channel could be calculated from the following equation (Hille 1968):

$$R_c = \left(l + \frac{\pi r}{2} \right) \frac{\rho}{\pi r^2}$$

This equation relates the channel resistance (R_c , which is the reciprocal of the conductance) to the pore radius (r), the pore length (l), and the conductivity of the solution inside the channel (ρ). Using previously proposed values (Cruickshank et al. 1997) for channel length ($l = 42 \text{ \AA}$) and recording solution conductivity ($\rho = 0.5 \text{ \Omega m}$), the pore radius of a pentameric channel of 3.0 nS conductance would be 18.2 \AA . If we increase this radius by a factor of 1.2, the conductance of the hexameric channel would be $\sim 4.0 \text{ nS}$. Such an increase in conductance would have been noticed with our experimental set-up but was not observed. We therefore propose that the hexameric construct also forms a channel with a pentameric structure, that is, with one subunit not participating in the channel. Along the same line of reasoning, it can be concluded that the dimeric, trimeric, and tetrameric constructs must be composed of the number of polypeptide chains indicated in Figure 5, B and C.

Implications of linker length for the structure of the pentameric construct

In the covalently-linked MscL constructs, the C terminus of MscL 1–4 is conjugated to the N terminus of the next MscL by a linker of only two amino acids. As all constructs were able to form functional channels, this implies that in the open and closed conformations, the N and C termini of the protein can be close together. This is in contrast to the situation in the three-dimensional structure of MscL from *M. tuberculosis*, where the N and C termini are $\sim 35 \text{ \AA}$ apart (Chang et al. 1998). In *E. coli* this distance may be different, as MscL from *E. coli* is 15 amino acids shorter than MscL from *M. tuberculosis*. Studies in which the C-terminal helices of MscL from *E. coli* were subjected to cysteine cross-linking with the channel in the open or closed state (Anishkin et al. 2003) showed that these helices form a rigid helix bundle that is probably directed toward the cytoplasm. It is also known that MscL tolerates relatively large truncations of up to 27 residues from the C terminus (Blount et al. 1996b), and separately expressed N- and C-terminal halves of the protein still form functional channels (Park

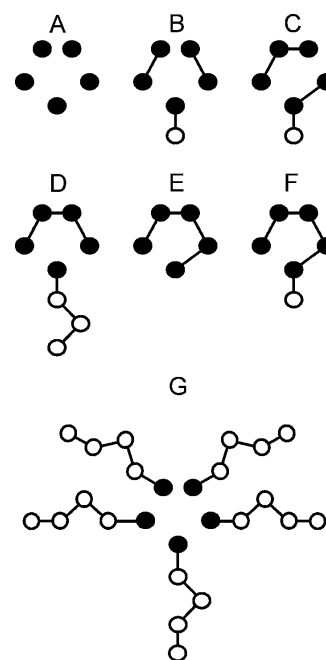


Figure 5. Schematic depiction of the possible ways the covalently linked multimeric MscL constructs might form functional channels with a “pentameric” structure. Each filled circle indicates a subunit participating in the formation of an active channel. The open circles indicate nonparticipating subunits. Dimer and trimer schemes are as suggested previously (Sukharev et al. 1999). (A) monomeric construct, (B) dimeric tandem construct, (C) trimeric tandem construct, (D) tetrameric tandem construct, (E) pentameric tandem construct, (F) hexameric tandem construct, and (G) alternative structure formed from pentameric tandem constructs.

et al. 2004). This, together with our results, suggests that the position and structure of the C terminus is not very critical for the formation of functional channels.

In summary, we have shown that covalently-linked multi-meric constructs of up to six MscL proteins are capable of forming functional pores and that the pentameric construct resembles wild-type MscL in its channel properties. We conclude that the oligomeric state of MscL from *E. coli* is pentameric. The pentameric gene cassette with unique restriction sites between the individual genes allows easy exchange of wild-type for mutant alleles. The covalent pentameric construct represents an important tool for analyzing the effect on channel function, of single and multiple amino acid substitutions per pore unit, rather than per subunit.

Materials and methods

Strains and growth conditions

All experiments were carried out with *E. coli* PB104 (*recA*[−] and $\Delta mscL::Cm^{res}$) as a host for the recombinant plasmids (Ou et al. 1998). Cells were grown under aerating conditions at 37°C in Luria Broth, supplemented with 100 µg/mL ampicillin and 30 µg/mL chloramphenicol. To search for optimal expression conditions of *mscL*, temperatures of 25°C and 30°C and different L-arabinose concentrations were tested. For growth on solid medium, 1.5% (w/v) agar was added to the broth.

Construction of plasmids

The plasmids used in this study are listed in Table 1. The tandem constructs were made by ligating *mscL* genes one by one into the pBADmyc-HisB vector as shown in Figure 1A. The individual genes were amplified by PCR, using the primers listed in Table 2. During PCR amplification, restriction sites were created upstream and downstream of the *mscL* gene for directional insertion and positioning of the DNA into the vector. PCR amplifications, using Expand High-Fidelity DNA polymerase (Roche Applied Science) were performed

according to the manufacturer's instructions. The DNA was amplified by PCR using pB10bMscL as template, an annealing temperature of 53°C, and an elongation time of 60 sec.

To the first inserted gene (*mscL5*), a sequence coding for a C-terminal 10-histidine tag was added in a two-step PCR. Primers MscL5 Fw and MscL5-7H Rev were used to introduce a seven-histidine tag. In a second step, using the product of the first amplification as template and primers MscL5 Fw and MscL5-10H Rev, another three histidines, a stop codon, and a restriction site for further cloning were introduced. The gene was then inserted into the pBADmyc-HisB plasmid, using EcoRI and HindIII restriction enzymes and T4 ligase (Roche Applied Science) according to the manufacturer's instructions, forming pBADMscL5. *mscL4* was inserted into pBADMscL5 using the KpnI and EcoRI restriction sites, resulting in pBADMscL45. Next *mscL3*, *mscL2*, and *mscL1* were inserted sequentially using the restriction sites shown in Figure 1A and in Table 2, resulting in pBADMscL345, pBADMscL2345, and pBADMscL12345, respectively. To introduce the sixth *mscL* gene into pBADMscL12345, the fifth gene (*mscL1*) was replaced by a two-gene fusion (*mscL1AB*) constructed in pGFPCR. First, *mscL1B* was inserted into pGFPCR, using XhoI and SacI restriction sites, and then *mscL1A* was inserted, using NcoI and XhoI restriction sites, resulting in pGFPCRMscL1AB.

The oligomeric tandem constructs were used to transform *E. coli* PB104, and after isolation and purification of the plasmids (Qiagen), the DNA sequence was analyzed to confirm fidelity. Sequencing showed that *mscL5* contained a silent mutation. In addition, *mscL4* contained a methionine to leucine substitution at position 1 of the MscL coding region.

Expression

E. coli PB104 containing the plasmids with the *mscL* tandem constructs were grown in LB medium to an OD₆₀₀ of 0.8, after which expression was triggered by addition of L-arabinose (0.0025%–0.25% w/v) and induction for different time periods as suggested in Guzman et al. (1995). Inside-out membrane vesicles were prepared by lysing the bacteria (20 mg/mL protein) by a twofold passage through a French pressure cell at 10,000 psi and removal of unlysed cells and cell wall debris by centrifugation at 30,000g. The membrane vesicles were washed once by centrifugation at 150,000g and then resuspended in 50

Table 1. List of plasmids used in this study and their characteristics

Plasmid	Relevant characteristics	Reference
pB10b	pBR322 ori; lacUV5 promoter; XbaI, XhoI in multiple cloning site, Amp ^{res}	Ou et al. 1998
pB10bmscL	pB10b with <i>mscL</i> , with a C-terminal 6 histidine tag inserted in XbaI, XhoI	Ou et al. 1998
pBADmyc-HisB	pBR322 ori; P _{BAD} promoter; NcoI, HindIII in multiple cloning site, Amp ^{res}	Invitrogen
pGFPCR	pUC ori; GFPuv, NcoI, XhoI and SacI in multiple cloning site, Amp ^{res}	Cormack et al. 1998
pBADMscL5	pBADmyc-HisB with <i>mscL5-10H</i> inserted in EcoRI, HindIII	This work
pBADMscL45	pBADMscL5 with <i>mscL4</i> inserted in KpnI, EcoRI	This work
pBADMscL345	pBADMscL4,5 with <i>mscL3</i> inserted in BglII, KpnI	This work
pBADMscL2345	pBADMscL3,4,5 with <i>mscL2</i> inserted in BglII, SacI	This work
pBADMscL12345	pBADMscL2,3,4,5 with <i>mscL1</i> inserted in SacI, NcoI	This work
pBADMscL1AB2345	pBADMscL1,2,3,4,5 with <i>mscL1</i> replaced by <i>mscL1AB</i> from pGFPCRMscL1AB using SacI, NcoI	This work
pGFPCRMscL1A	pGFPCR with <i>mscL1A</i> inserted in XhoI, SacI	This work
pGFPCRMscL1AB	pGFPCRMscL1A with <i>mscL1B</i> inserted in NcoI, XhoI	This work

Table 2. Primers and their relevant characteristics

Subunit	Primers	Sequence	Site
MscL5	Fw	5'-ATATATGAATTCATGAGCATTATTAAGAATTCGCG	EcoRI
MscL5-7H	Rev	5'-GTGGTGATGGTGATGGTGATGAGAGCGGTTATTCTGCTCTTTCAGC	
MscL5	Fw	5'-ATATATGAATTCATGAGCATTATTAAGAATTCGCG	HindIII
MscL5-10H	Rev	5'-ATATATAAGCTTTTAATGGTGATGGTGATGGTGATGGTGATGGTGATGAGAGC	
MscL4	Fw	5'-ATATATGGTACCAGAGCATTATTAAGAATTCGCG	KpnI
MscL4	Rev	5'-ATATATGAATTCAGAGCGGTTATTCTGCTCTTTCAGC	EcoRI
MscL3	Fw	5'-ATATATAGATCTATGAGCATTATTAAGAATTCGCG	BglII
MscL3	Rev	5'-ATATATGGTACCAGAGCGGTTATTCTGCTCTTTCAGC	KpnI
MscL2	Fw	5'-ATATATGAGCTCATGAGCATTATTAAGAATTCGCG	SacI
MscL2	Rev	5'-ATATATAGATCTAGAGCGGTTATTCTGCTCTTTCAGC	BglII
MscL1	Fw	5'-ATATATCCATGGGCATTATTAAGAATTCGCG	NcoI
MscL1	Rev	5'-ATATATGAGCTCAGAGCGGTTATTCTGCTCTTTCAGC	SacI
MscL1A	Fw	5'-ATATATCTCGAGATGAGCATTATTAAGAATTCGCG	XhoI
MscL1	Rev	5'-ATATATGAGCTCAGAGCGGTTATTCTGCTCTTTCAGC	SacI
MscL1	Fw	5'-ATATATCCATGGGCATTATTAAGAATTCGCG	NcoI
MscL1B	Rev	5'-ATATATCTCGAGAGAGCGGTTATTCTGCTCTTTCAGC	XhoI

mM KPi (pH 6.5). Aliquots of 0.5 mL were frozen in liquid nitrogen and stored at -80°C until use.

Spheroplast preparation

Giant spheroplasts were prepared from *E. coli* PB104, carrying the plasmids with the *mscL* oligomeric tandem constructs, essentially as described (Blount et al. 1999), except that protein synthesis was triggered using 0.25% L-arabinose for 30 min.

Electrophysiology

Experiments were performed as described previously (Blount et al. 1999; Folgering et al. 2005). Samples of 1–5 μL of spheroplast (0.2–0.8 mg/mL total protein) were transferred to a sample chamber containing a ground electrode and 300 μL of patch clamp buffer: 5 mM HEPES (pH 7.2), 200 mM KCl, 90 mM MgCl_2 , plus 10 mM CaCl_2 . Channel activity was recorded using an Axopatch 200A amplifier together with a digital converter and Axoscope software (Axon Instruments). Data were acquired at a sampling rate of 33 kHz and filtered at 10 kHz. Offline analysis was performed using Clampfit 8.0 software (Axon Instruments). Traces shown in Figure 3 were filtered with the low-pass Boxcar filter at smoothing point 7. Pressure ratios for activation of MscL relative to the activation of MscS were determined, as described previously (Blount et al. 1996b, 1999). Briefly, the pressure for MscS gating was defined as the pressure required for the simultaneous opening of two or more channels. The MscL opening threshold was defined as the pressure at which the first full opening was observed.

Miscellaneous

Purified proteins were analyzed on 5%–20% polyacrylamide gradient SDS–PAGE (Laemmli et al. 1970). Protein expression levels in membrane vesicles were analyzed by immunodetection, using antibodies raised against the his-tag (Amersham) and the Western-light chemoluminescence detection kit (Tropix Inc.).

Acknowledgments

We thank E.R. Geertsma and the channel protein group at the BioMaDe Technology Foundation for helpful discussions, and MSC^{plus} for financial support.

References

- Anishkin, A., Gendel, V., Sharifi, N.A., Chiang, C.S., Shirinian, L., Guy, H.R., and Sukharev, S. 2003. On the conformation of the COOH-terminal domain of the large mechanosensitive channel MscL. *J. Gen. Physiol.* **121**: 227–244.
- Becker, C.F., Strop, P., Bass, R.B., Hansen, K.C., Locher, K.P., Ren, G., Yeager, M., Rees, D.C., and Kochendoerfer, G.G. 2004. Conversion of a mechanosensitive channel protein from a membrane-embedded to a water-soluble form by covalent modification with amphiphiles. *J. Mol. Biol.* **343**: 747–758.
- Blount, P., Sukharev, S.I., Moe, P.C., Schroeder, M.J., Guy, H.R., and Kung, C. 1996a. Membrane topology and multimeric structure of a mechanosensitive channel protein of *Escherichia coli*. *EMBO J.* **15**: 4798–4805.
- Blount, P., Sukharev, S.I., Schroeder, M.J., Nagle, S.K., and Kung, C. 1996b. Single residue substitutions that change the gating properties of a mechanosensitive channel in *Escherichia coli*. *Proc. Natl. Acad. Sci.* **93**: 11652–11657.
- Blount, P., Sukharev, S.I., Moe, P.C., Martinac, B., and Kung, C. 1999. Mechanosensitive channels of bacteria. *Methods Enzymol.* **294**: 458–482.
- Chang, G., Spencer, R.H., Lee, A.T., Barclay, M.T., and Rees, D.C. 1998. Structure of the MscL homolog from *Mycobacterium tuberculosis*: A gated mechanosensitive ion channel. *Science* **282**: 2220–2226.
- Cormack, R.S., Hahlbrock, K., and Somssich, I.E. 1998. Isolation of putative plant transcriptional coactivators using a modified two-hybrid system incorporating a GFP reporter gene. *Plant J.* **14**: 685–692.
- Cruickshank, C.C., Minchin, R.F., Le Dain, A.C., and Martinac, B. 1997. Estimation of the pore size of the large-conductance mechanosensitive ion channel of *Escherichia coli*. *Biophys. J.* **73**: 1925–1931.
- Folgering, J.H., Moe, P.C., Schuurman-Wolters, G.K., Blount, P., and Poolman, B. 2005. *Lactococcus lactis* uses MscL as its principal mechanosensitive channel. *J. Biol. Chem.* **280**: 8784–8792.
- Guzman, L.M., Belin, D., Carson, M.J., and Beckwith, J. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J. Bacteriol.* **177**: 4121–4130.
- Hase, C.C., Le Dain, A.C., and Martinac, B. 1995. Purification and functional reconstitution of the recombinant large mechanosensitive ion channel (MscL) of *Escherichia coli*. *J. Biol. Chem.* **270**: 18329–18334.

- . 1997. Molecular dissection of the large mechanosensitive ion channel (MscL) of *E. coli*: Mutants with altered channel gating and pressure sensitivity. *J. Membr. Biol.* **157**: 17–25.
- Hille, B. 1968. Pharmacological modifications of the sodium channels of frog nerve. *J. Gen. Physiol.* **51**: 199–219.
- Laemmli, U.K., Beguin, F., and Gujer-Kellenberger, G. 1970. A factor preventing the major head protein of bacteriophage T4 from random aggregation. *J. Mol. Biol.* **47**: 69–85.
- Liman, E.R., Tytgat, J., and Hess, P. 1992. Subunit stoichiometry of a mammalian K^+ channel determined by construction of multimeric cDNAs. *Neuron* **9**: 861–871.
- Ou, X., Blount, P., Hoffman, R.J., and Kung, C. 1998. One face of a transmembrane helix is crucial in mechanosensitive channel gating. *Proc. Natl. Acad. Sci.* **95**: 11471–11475.
- Park, K.H., Berrier, C., Martinac, B., and Ghazi, A. 2004. Purification and functional reconstitution of N- and C-halves of the MscL channel. *Biophys. J.* **86**: 2129–2136.
- Poolman, B., Blount, P., Folgering, J.H.A., Friesen, R.H., Moe, P.C., and van der Heide, T. 2002. How do membrane proteins sense water stress? *Mol. Microbiol.* **44**: 889–902.
- Saint, N., Lacapere, J.J., Gu, L.Q., Ghazi, A., Martinac, B., and Rigaud, J.L. 1998. A hexameric transmembrane pore revealed by two-dimensional crystallization of the large mechanosensitive ion channel (MscL) of *Escherichia coli*. *J. Biol. Chem.* **273**: 14667–14670.
- Stahlberg, H., Muller, D.J., Suda, K., Fotiadis, D., Engel, A., Meier, T., Matthey, U., and Dimroth, P. 2001. Bacterial Na^+ -ATP synthase has an undecameric rotor. *EMBO Rep.* **2**: 229–233.
- Sugawara-Tomita, N., Tomita, T., and Kamio, Y. 2002. Stochastic assembly of two-component staphylococcal γ -hemolysin into heteroheptameric transmembrane pores with alternate subunit arrangements in ratios of 3:4 and 4:3. *J. Bacteriol.* **184**: 4747–4756.
- Sukharev, S.I., Blount, P., Martinac, B., Blattner, F.R., and Kung, C. 1994. A large-conductance mechanosensitive channel in *E. coli* encoded by *mscL* alone. *Nature* **368**: 265–268.
- Sukharev, S.I., Schroeder, M.J., and McCaslin, D.R. 1999. Stoichiometry of the large conductance bacterial mechanosensitive channel of *E. coli*. A biochemical study. *J. Membr. Biol.* **171**: 183–193.
- Yoshimura, K., Batiza, A., Schroeder, M., Blount, P., and Kung, C. 1999. Hydrophilicity of a single residue within MscL correlates with increased channel mechanosensitivity. *Biophys. J.* **77**: 1960–1972.